Acta Biochim Biophys Sin 2012, 44: 703–711 | © The Author 2012. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gms052.
Advance Access Publication 26 June 2012

Original Article

Effects of macromolecular crowding on the structural stability of human α-lactalbumin

De-Lin Zhang, Ling-Jia Wu, Jie Chen, and Yi Liang*

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China
*Correspondence address. Tel: +86-27-68754902; Fax: +86-27-68754902; E-mail: liangyi@whu.edu.cn

The folding of protein, an important process for protein to fulfill normal functions, takes place in crowded physiological environments. α-Lactalbumin, as a model system for protein-folding studies, has been used extensively because it can form stable molten globule states under a range of conditions. Here we report that the crowding agents Ficoll 70, dextran 70, and polyethylene glycol (PEG) 2000 have different effects on the structural stability of human α-lactalbumin (HLA) represented by the transition to a molten globule state: dextran 70 dramatically enhances the thermal stability of Ca^{2+}-depleted HLA (apo-HLA) and Ficoll 70 enhances the thermal stability of apo-HLA to some extent, while PEG 2000 significantly decreases the thermal stability of apo-HLA. Ficoll 70 and dextran 70 have no obvious effects on trypsin degradation of apo-HLA but PEG 2000 accelerates apo-HLA degradation by trypsin and destabilizes the native conformation of apo-HLA. Furthermore, no interaction is observed between apo-HLA and Ficoll 70 or dextran 70, but a weak, non-specific interaction between the apo form of the protein and PEG 2000 is detected, and such a weak, non-specific interaction could overcome the excluded-volume effect of PEG 2000. Our data are consistent with the results of protein stability studies in cells and suggest that stabilizing excluded-volume effects of crowding agents can be ameliorated by non-specific interactions between proteins and crowders.

Keywords human α-lactalbumin; macromolecular crowding; protein stability; fluorescence spectroscopy; circular dichroism; trypsin digestion

Received: December 6, 2011 Accepted: April 27, 2012

Introduction

The folding of proteins, an important process for the proteins to fulfill their normal functions, takes place in crowded physiological environments [1–3]. The effect of macromolecular crowding on protein folding and stability is a double-edged sword [4,5]. On the one hand, macromolecular crowding destabilizes expanded unfolded protein conformations. These expanded protein molecules collapse to more compact conformations, and thus both protein folding and protein stability against thermal and chemical denaturation as well as mechanical force are enhanced [6–19]. On the other hand, macromolecular crowding enhances undesirable aggregation of partially unfolded proteins, thereby not favoring protein folding and stability [20,21].

α-Lactalbumin has been used extensively as a model system for protein-folding studies because it can form stable molten globule states under a range of conditions [22–24]. α-Lactalbumin has an amino acid sequence and conformation comparable with that of C-type lysozymes, but contains a Ca^{2+}-binding site which stabilizes the native conformation of α-lactalbumin [24]. Under destabilizing conditions such as low pH, the addition of moderate concentration of guanidine hydrochloride, and high temperature, the apo form of α-lactalbumin (Ca^{2+}-depleted α-lactalbumin) forms stable molten globule states [25]. The molten globule state is characterized by the absence of long-lived tertiary structure, but still contains a high degree of secondary structure with a radius of gyration only 10% larger than the native state [26–29]. The molten globule state of α-lactalbumin has attracted considerable interest because it could act as an important component causing apoptosis of tumor cells [30–32].

Ficoll 70 and dextran 70 are widely accepted as perfect models for the principal crowding components in living cells where the folding of proteins takes place, because their interactions with proteins can be described using pure excluded-volume models [3,33]. By contrast, polyethylene glycol (PEG) is another kind of crowding agent, whose interactions with proteins cannot be described quantitatively in terms of excluded volume alone [3]. The excluded volume effects of high-molecular-mass PEG are more effective than those of polysaccharide crowding agents, and an attractive interaction between PEG and non-polar or hydrophobic side chains on the protein surface is accompanied [3,10]. In this study, we demonstrated for the first time that the three crowding agents Ficoll 70, dextran 70,

and PEG 2000 had different effects on the structural stability of human α-lactalbumin (HLA) represented by the transition into a molten globule state: dextran 70 dramatically enhanced the structural stability of Ca^{2+}-depleted HLA (apo-HLA) and Ficoll 70 enhanced that of apo-HLA to some extent, whereas PEG 2000 significantly decreased the structural stability of apo-HLA. Furthermore, no interaction between apo-HLA and Ficoll 70 or dextran 70 but a weak, non-specific interaction between apo-HLA and PEG 2000 was observed. Such a weak, non-specific interaction could overcome the excluded-volume effect of PEG 2000, thereby decreasing the structural stability of apo-HLA.

Materials and Methods

Materials

HLA was prepared and purified as described previously [34]. Purified HLA was homogenous on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The $A^{1\%}_{1cm}$ value of 18.2 [35,36] at 280 nm was used for protein concentration measurements. The crowding agents, Ficoll 70, dextran 70, and PEG 2000/20000, were obtained from Sigma (Sigma-Aldrich, St Louis, USA). Dithiothreitol was a Biomol product (Biomol Co., Hamburg, Germany). All other chemicals used were made in China and of analytical grade. All reagent solutions used were prepared in 10 mM NaH_2PO_4–Na_2HPO_4 buffer containing 100 mM NaCl and 1 mM EGTA (pH 7.0) unless specified otherwise.

Intrinsic fluorescence spectroscopy

Intrinsic fluorescence measurements at a protein concentration of 10 μM were performed using an excitation wavelength of 280 nm and emission was collected between 300 and 400 nm. The excitation and emission slits were 10 and 4 nm, respectively, and the scan speed was 100 nm/min. Measurements were performed at 20.0–60.0°C.

Circular dichroism measurements

Circular dichroism (CD) spectra were obtained by using a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan) with a thermostated cell holder. Quartz cells with a 1-mm light path length and a 10-mm light path length were used for measurements in the far-ultraviolet (UV) regions and the near-UV regions, respectively. Spectra were recorded from 195 to 250 nm for far-UV CD and from 250 to 300 nm for near-UV CD, respectively. The final concentrations of apo-HLA were kept at 10 μM for far-UV CD and 70 μM for near-UV CD, respectively. The averaged spectra of several scans were corrected relative to the buffer blank. The mean residue ellipticity $[\theta]$ (degree $cm^2 \bullet dmol^{-1}$) was calculated using the formula $[\theta] = (\theta_{obs}/10)(MRW/lc)$, where θ_{obs} is the observed ellipticity in degrees, MRW the mean residue molecular weight (115.3 Da for apo-HLA), l the path length in centimeters, and c the protein concentration in grams per milliliter. Measurements were made at 25.0 or 55.0°C.

Thermal unfolding measurements

Thermal unfolding of apo-HLA at pH 7.0, represented by the transition to a molten globule state, was achieved by intrinsic fluorescence spectroscopy, by increasing the temperature from 20.0 to 60.0°C. The denaturation of apo-HLA under such conditions was reversible. The protein was excited at 280 nm and emission data were collected at 365 nm with a heating rate of 3°C/min. The accurate temperature of the sample was recorded by inserting a K-type tip temperature meter into a 1-cm thermostated quartz fluorescence cuvette containing apo-HLA solution. The thermal unfolding curves of apo-HLA were globally fit to the following equation:

$$F_{obs} = \frac{[(a_n + b_n T) + (a_u + b_u T)e^{-\Delta G_u(T)/RT}]}{[1 + e^{-\Delta G_u(T)/RT}]} \quad (1)$$

where F_{obs} is the observed fluorescence intensity at a given temperature, a and b are the intercept and slope, respectively, of pre- (subscript n) and post-transition (subscript u) baselines, R denotes the molar gas constant and T the absolute temperature. $\Delta G_u(T)$ is the standard Gibbs free energy change upon apo-HLA unfolding and is defined as

$$\Delta G_u(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T\ln(T/T_m)] \quad (2)$$

where ΔH_m and ΔC_p are the changes in enthalpy and heat capacity associated with apo-HLA unfolding, respectively, and the melting temperature (T_m) is the mid-point of the thermal unfolding curve, the temperature at which $\Delta G_u(T) = 0$.

The fraction of unfolded apo-HLA, $f(U)$, versus temperature was calculated according to the following equation:

$$f(U) = \frac{F_{obs} - a_n - b_n T}{a_u + b_u T - a_n - b_n T} \quad (3)$$

Trypsin proteolysis

For the digestion experiments of apo-HLA, samples were treated with trypsin at apo-HLA : trypsin mass ratio 10 : 1 in the absence and in the presence of a crowding agent (100 g/L Ficoll 70, 100 g/L dextran 70, or 2–100 g/L PEG 2000/20000). After incubation in 10 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) at 25.0°C for 10 min, 10 μl of

each sample was subjected to 13.5% SDS-PAGE. Staining of gels was with Coomassie Blue.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments on the binding of dextran 70, Ficoll 70, or PEG 2000 to apo-HLA were carried out at 37.0 °C and pH 7.0 using an iTC_{200} titration calorimetry (MicroCal, Northampton, USA). A solution of 14 μM apo-HLA was loaded into the sample cell (200 μl), and a solution of 0.714 mM dextran 70, 0.714 mM Ficoll 70, or 25.0 mM PEG 2000 was placed in the injection syringe (40 μl). The first injection (0.5 μl) was followed by 18 injections of 2 μl. Dilution heats of dextran 70, Ficoll 70, or PEG 2000 were measured by injecting dextran 70, Ficoll 70, or PEG 2000 solution into buffer alone and were subtracted from the experimental curves prior to data analysis. The stirring rate was 600 rpm. MicroCal ORIGIN software supplied with the instrument was used to determine the site binding model that gave a good fit (low χ^2 value) to the resulting data.

Results

Molten globule state of apo-HLA

The pH dependence and temperature dependence of structural changes of apo-HLA was characterized in **Fig. 1**, using CD and fluorescence spectroscopy. Curves a, b, c, and d in **Fig. 1** represent the spectra of apo-HLA at pH 7.0 and 25.0°C, pH 7.0 and 55°C (thermal-induced molten globule state), pH 3.0 and 25.0°C (acid-induced molten globule state), and pH 7.0 and 25.0°C in the presence of 7.0 M guanidine hydrochloride (the unfolded state), respectively. As shown in **Fig. 1**, the apo state of HLA had an intrinsic fluorescence emission maximum of ~336 nm when excited at 280 nm, and was characterized with well-defined secondary structure (α-helix) and well-defined tertiary structure. The tryptophan and tyrosine emission maximum of the protein was red-shifted, reaching 346 nm for the thermal-induced molten globule state, to 349 nm for the acid-induced molten globule state, and to 356 nm for the unfolded state [**Fig. 1(A)**]. This indicates that in such states, the tryptophan and tyrosine residues are gradually exposed to a more hydrophilic environment. As shown in **Fig. 1(B,C)**, both thermal-induced and acid-induced molten globule states of apo-HLA were characterized with stabilized secondary structure but almost entirely without rigid tertiary structure. There is an almost complete loss of well-defined secondary structure and rigid tertiary structure for the unfolded state of apo-HLA.

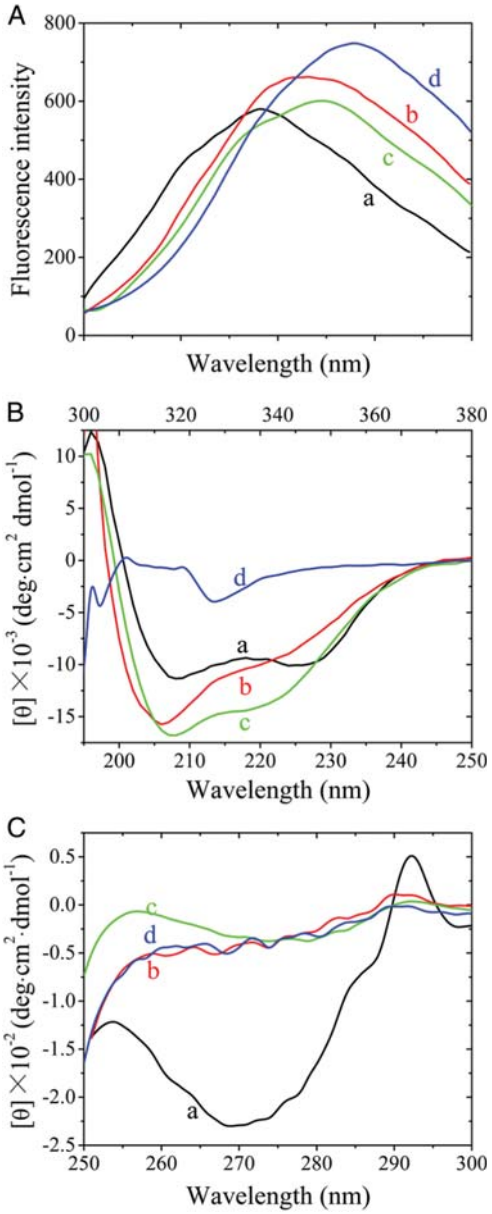

Figure 1 Molten globule state of apo-HLA detected by intrinsic fluorescence emission, far-UV CD, and near-UV CD (A) Intrinsic fluorescence emission spectra of apo-HLA. (B) Far-UV CD spectra of apo-HLA. (C) Near-UV CD spectra of apo-HLA. Curves a, b, c, and d represent the spectra of apo-HLA at pH 7.0 and 25.0°C, pH 7.0 and 55.0°C (thermal-induced molten globule), pH 3.0 and 25°C (acid-induced molten globule), and pH 7.0 and 25.0°C in the presence of 7.0 M guanidine hydrochloride (unfolded state), respectively.

Effects of macromolecular crowding agents on the thermal stability of apo-HLA

As mentioned above, we observed a two-state transition between the apo state and the molten globule state for thermal-induced unfolding of apo-HLA at pH 7.0, similar to a recent report concerning thermal-induced unfolding of bovine apo-α-lactalbumin at pH 7 [37]. **Figure 2(A)** shows fluorescence spectra of apo-HLA unfolded thermally in the

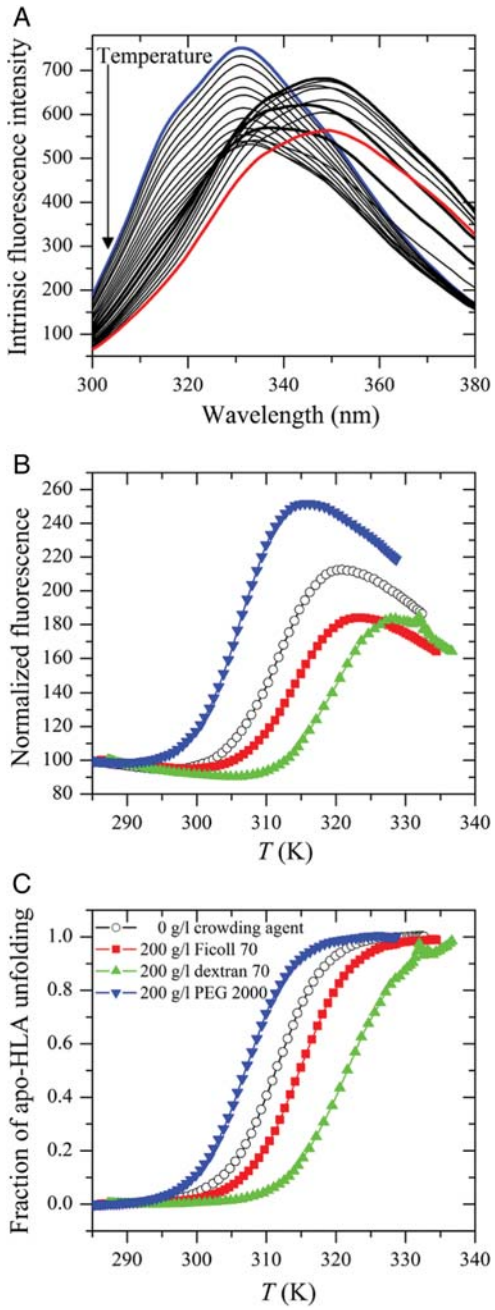

Figure 2 Effects of macromolecular crowding agents on the thermal stability of apo-HLA represented by the transition to a molten globule state monitored by intrinsic fluorescence spectroscopy (A) Fluorescence spectra of 10 μM apo-HLA unfolded thermally at pH 7.0 in the absence of crowding agents. The arrow represents the incubation temperature increased gradually from 20.0°C (the top, blue) to 60.0°C (the bottom, red). (B) Diagram of normalized fluorescence intensity at 365 nm versus temperature. (C) Diagram of the fraction of unfolded apo-HLA versus temperature. Diagram C was achieved by fitting the data of diagram B to equation (3). Thermal unfolding curves of apo-HLA at pH 7.0 in the absence of a crowding agent (open circle), in the presence of 200 g/L Ficoll 70 (solid square), in the presence of 200 g/L dextran 70 (solid triangle), and in the presence of 200 g/L PEG 2000 (solid inverse triangle).

absence of crowding agents. As shown in **Fig. 2(B)**, when the incubation temperature increased gradually from 20.0 to 60.0°C, a gradual decrease in fluorescence intensity took place at the first stage, a significant increase in fluorescence intensity together with a red shift of intrinsic fluorescence emission maximum occurred at the second stage, and a gradual decrease in fluorescence intensity took place once again at the final stage. Here we studied the effects of macromolecular crowding agents on the thermal stability of apo-HLA. We collected the data of fluorescence intensity at 365 nm with a heating rate of 3°C/min and obtained the thermal unfolding curves of apo-HLA in the absence of a crowding agent and in the presence of 200 g/L Ficoll 70, dextran 70, or PEG 2000 [**Fig. 2(B,C)**], and in the presence of 50, 100, 200, and 300 g/L one of these crowding agents (**Fig. 3**). The melting temperature of apo-HLA at pH 7.0 was obtained by best fitting the data of intrinsic fluorescence in **Figs. 2** and **3** to a two-state model represented by equations (1) and (2). The calculated best-fit curves and the experimental results are in good agreement within the experimental errors. Thermal stabilities of apo-HLA in the presence of different concentrations of crowding agents and in dilute solutions are summarized in **Table 1**. Apo-HLA had a melting temperature of 312.8 K (39.7°C) in dilute solutions (**Table 1**). We obtained a similar value of T_m of apo-HLA in the absence of a crowding agent when we fit the data of fluorescence intensity at 350 nm (or 340 nm) to equations (1) and (2) (data not shown). As shown in **Table 1**, the melting temperature of apo-HLA in the presence of 300 g/L dextran 70 (52.3°C) and in the presence of 300 g/L Ficoll 70 (43.5°C) increased 12.6 and 3.8°C, respectively, but T_m of apo-HLA in the presence of 300 g/L PEG 2000 (33.6°C) and in the presence of 300 g/L PEG 20000 (35.6°C) decreased 6.1 and 4.1°C, respectively, compared with that in dilute solutions. On average, the thermal midpoint (the melting temperature) was increased by 8 and 3°C in the presence of dextran 70 and Ficoll 70, respectively, but decreased by 5 and 4°C in the presence of PEG 2000 and PEG 20000, respectively, as compared with in buffer. Given the model used to analyze the data, it is possible to report differences in the free energy changes (ΔG), which would be more useful for purposes of comparisons with theory and other experiments [6,9,12–14]. As shown in **Fig. 4**, at the same temperature, the free energy change of apo-HLA in the presence of 200 g/L dextran 70 or 200 g/L Ficoll 70 increased remarkably, but ΔG of apo-HLA in the presence of 200 g/L PEG 2000 or 200 g/L PEG 20000 decreased noticeably, compared with that in dilute solutions. Clearly, the crowding agents Ficoll 70, dextran 70, and PEG 2000/20000 had different effects on the structural stability of HLA represented by the transition to a molten globule state: dextran 70 dramatically enhanced the thermal stability of apo-HLA and Ficoll 70 enhanced the thermal stability of apo-HLA to some extent, but PEG 2000/20000 significantly decreased the thermal stability of apo-HLA (**Table 1**, **Fig. 4**).

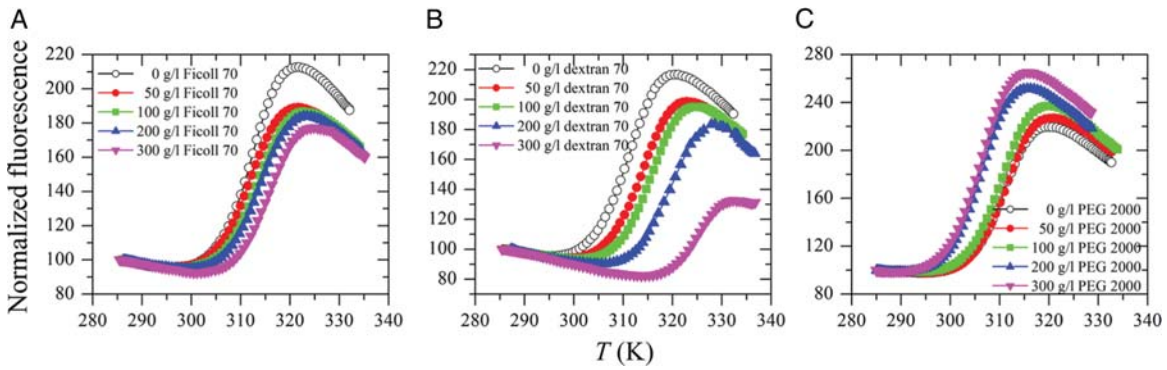

Figure 3 Effects of macromolecular crowding agents at different concentrations on the thermal stability of apo-HLA represented by the transition to a molten globule state monitored by intrinsic fluorescence spectroscopy Diagrams of normalized fluorescence intensity at 365 nm versus temperature in the presence of 0–300 g/L Ficoll 70 (A), in the presence of 0–300 g/L dextran 70 (B), and in the presence of 0–300 g/L PEG 2000 (C). Thermal unfolding curves of apo-HLA at pH 7.0 in the absence of a crowding agent (open circle), and in the presence of 50 g/L (solid circle), 100 g/L (solid square), 200 g/L (solid triangle), and 300 g/L (solid inverse triangle) crowding agent. The concentration of apo-HLA was 10 μM.

Table 1 Thermal stability of apo-HLA in the presence of different concentrations of crowding agents

Crowding agent concentration (g/L)	T_m (K)			
	Dextran 70	Ficoll 70	PEG 2000	PEG 20000
0	312.8 ± 0.1	312.8 ± 0.1	312.8 ± 0.1	312.8 ± 0.1
100	316.2 ± 0.1	314.6 ± 0.1	311.1 ± 0.1	310.9 ± 0.1
200	321.6 ± 0.2	314.8 ± 0.0	307.0 ± 0.1	310.6 ± 0.1
300	325.4 ± 0.2	316.6 ± 0.1	306.7 ± 0.1	308.7 ± 0.1

The melting temperature (T_m) of apo-HLA in the presence of dextran 70, Ficoll 70, or PEG 2000 was obtained by fitting the data of intrinsic fluorescence in **Figs. 2** and **3** to equations (1) and (2). Errors shown are standard errors of the mean.

Effects of macromolecular crowding on apo-HLA degradation by trypsin

Next, we examined the structural stability of apo-HLA at pH 7.0 in the presence of one of the three crowding agents by using digestion with trypsin (**Fig. 5**). As shown in **Fig. 5(A)**, for apo-HLA incubated in the absence of a crowding agent, a protein band (thick band) corresponding to apo-HLA (the apo state) and another band (thin band) corresponding to a fragment of apo-HLA were observed when treated with trypsin. Similarly, a thick band corresponding to apo-HLA and a thin band corresponding to a fragment of apo-HLA were observed when apo-HLA was treated with trypsin in the presence of 100 g/L Ficoll 70 or 100 g/L dextran 70 [**Fig. 5(A)**]. However, as shown in **Fig. 5(B,C)**, for apo-HLA incubated in the presence of 100 g/L PEG 2000 and 100 g/L PEG 20000, respectively, only the peptide band corresponding to a fragment of apo-HLA was observed when treated with trypsin, and a protein band corresponding to apo-HLA almost disappeared. Furthermore, such a protein band turned out to be fainter when the concentration of PEG 2000 increased from 2 to 50 g/L [**Fig. 5(B)**]. Clearly, Ficoll 70 and dextran 70 had no obvious effects on trypsin degradation of apo-HLA but PEG 2000 and PEG 20000 accelerated apo-HLA degradation by trypsin and destabilized the native conformation of apo-HLA.

Binding of dextran 70, Ficoll 70, or PEG 2000 to apo-HLA

ITC provides a direct route to the complete thermodynamic characterization of non-covalent, equilibrium interactions [38–43]. Therefore, we finally used ITC to measure the binding affinity of dextran 70, Ficoll 70, or PEG 2000 to apo-HLA. ITC profiles for the binding of dextran 70, Ficoll 70, or PEG 2000 to apo-HLA at 37.0°C are shown in **Fig. 6**. The top panels in **Fig. 6** representatively show raw ITC curves resulting from the injections of dextran 70 [**Fig. 6(A)**], Ficoll 70 [**Fig. 6(B)**], or PEG 2000 [**Fig. 6(C)**] into a solution of apo-HLA. The middle panels in **Fig. 6** show the background measurements in which dextran 70 [**Fig. 6(D)**], Ficoll 70 [**Fig. 6(E)**], or PEG 2000 [**Fig. 6(F)**] were injected into the buffer alone. The bottom panels in **Fig. 6** show the plot of the heat evolved per mole of crowder added, corrected for the heat of crowder dilution, against the molar ratio of dextran 70 [**Fig. 6(G)**], Ficoll 70 [**Fig. 6(H)**], or PEG 2000 [**Fig. 6(I)**] to apo-HLA. As shown in **Fig. 6**, the data from dextran 70 [**Fig. 6(G)**] and Ficoll 70 [**Fig. 6(H)**]) were too small to be fitted,

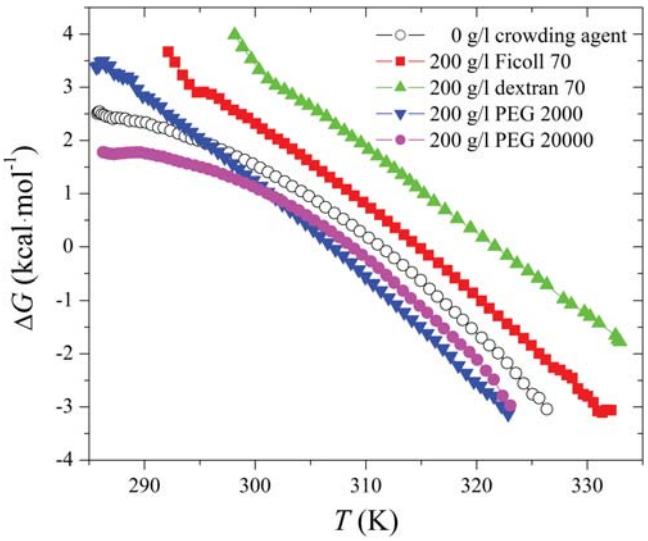

Figure 4 Effects of macromolecular crowding agents at different concentrations on the thermal stability of apo-HLA represented by the free energy changes monitored by intrinsic fluorescence spectroscopy Diagrams of the free energy changes versus temperature in the absence of a crowding agent (open circle), in the presence of 200 g/L Ficoll 70 (solid square), in the presence of 200 g/L dextran 70 (solid triangle), in the presence of 200 g/L PEG 2000 (solid inverse triangle), and in the presence of 200 g/L PEG 20000 (solid circle).

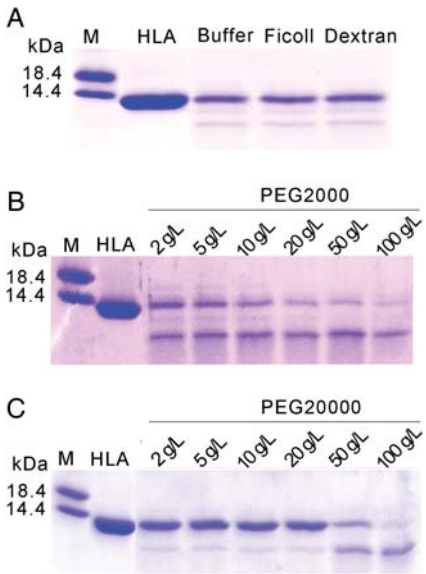

Figure 5 Effects of macromolecular crowding on apo-HLA degradation by trypsin at 25.0°C (A) Lanes Ficoll and dextran represent 70 μM apo-HLA treated by 0.10 mg/mL trypsin in the presence of 100 g/L Ficoll 70 and 100 g/L dextran 70, respectively. (B) The last six lanes represent 70 μM apo-HLA treated by 0.10 mg/mL trypsin in the presence of 2, 5, 10, 20, 50, and 100 g/L PEG 2000, respectively. (C) The last six lanes represent 70 μM apo-HLA treated by 0.10 mg/ml trypsin in the presence of 2, 5, 10, 20, 50, and 100 g/l PEG 20000, respectively. The buffer used was 10 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) containing 100 mM NaCl and 1 mM EGTA. M, protein molecular weight marker: β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). HLA, 70 μM apo-HLA alone, and lane buffer, 70 μM apo-HLA treated by 0.10 mg/ml trypsin in the absence of crowding agents.

indicating that no binding was observed in the conditions used. The best fit for the integrated heat data from PEG 2000 [**Fig. 6(I)**] was obtained using a three sequential binding sites model with the lowest χ^2 (35.6 cal mol^{-1}), yielding three binding constants of PEG 2000 to apo-HLA of 10^2–10^3 M^{-1} Clearly, no interaction between apo-HLA and Ficoll 70 (or dextran 70) was observed, but a weak, non-specific interaction between the apo form of the protein (a non-native form) and PEG 2000 was detected (**Fig. 6**), and such a weak, non-specific interaction could overcome the excluded-volume effect of PEG 2000 (**Table 1**). It should be pointed out that the signals are very weak, and they possibly come from the noises [**Fig. 6(I)**], because the profile in **Fig. 6(C)** (PEG 2000 to apo-HLA) is similar to that in **Fig. 6(F)** (PEG 2000 to buffer). In addition, the heat changes are extremely small (−0.03 to +0.01 kcal • mol^{-1}) [**Fig. 6(I)**], which are much smaller than the range of noise fluctuation (−0.2 to +0.2 kcal • mol^{-1}) [**Fig. 6(G,H)**]. Therefore, such a weak, non-specific interaction needs to be confirmed by other methods. These are planned for the future.

Discussion

The apo form of α-lactalbumin can form stable molten globule states under a range of conditions, including low pH, the addition of moderate concentration of guanidine hydrochloride, and high temperature [22–25]. In this study, we employed such kind of conditions (pH 7.0 and 55.0°C, or pH 3.0 and 25.0°C) to form thermal-induced molten globule or acid-induced molten globule of apo-HLA. We demonstrated that both thermal-induced and acid-induced molten globule states of apo-HLA were characterized with stabilized secondary structure but almost completely without rigid tertiary structure. Furthermore, the thermal-induced molten globule of apo-HLA was less unfolded than the acid-induced molten globule. The crystal structure of α-lactalbumin has revealed that the protein has 87 hydrogen bonds, 68 of them preserved in secondary structure and 19 preserved in tertiary structure [44]. Combining our data and the results from other groups [37,45], we conclude that the thermal-induced unfolding of apo-HLA follows a two-state model and that the thermal-induced molten globule of apo-HLA is possibly maintained by 68 hydrogen bonds preserved in its secondary structure.

The usual interpretation of macromolecular crowding effects is based on differences in the excluded volumes of alternate conformational states [4,6,9,11–16]. Because the molten globule of α-lactalbumin is only slightly larger than the native state (a difference of 10% in radius of gyration) [26–29], it is surprising that such a small change in volume would lead to what appears to be a substantial

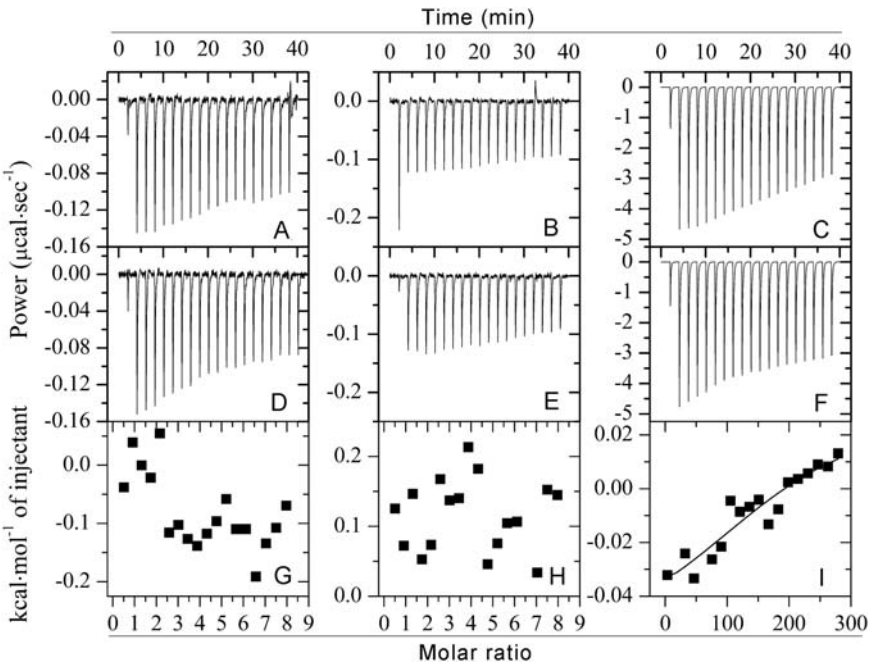

Figure 6 ITC profiles for the binding of dextran 70, Ficoll 70, or PEG 2000 to apo-HLA at 37.0°C The top panels represent the raw data for sequential 2-μl injections of 0.714 mM dextran 70 (A), 0.714 mM Ficoll 70 (B), or 25.0 mM PEG 2000 (C) into 14 μM apo-HLA, respectively, in 10 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) containing 100 mM NaCl and 1 mM EGTA. The middle panels represent the control experiments in which 0.714 mM dextran 70 (D) or Ficoll 70 (E), or 25.0 mM PEG 2000 (F) was injected into the buffer alone. The bottom panels (G, H, and I) show the plots of the heat evolved (kcal) per mole of dextran 70, Ficoll 70, or PEG 2000 added, corrected for the heat of dextran70, Ficoll 70, or PEG 2000 dilution, against the molar ratio of dextran 70, Ficoll 70, or PEG 2000 to apo-HLA. The data (solid square) were best fitted to a three sequential binding sites model and the solid lines represented the best fit (I). However, the data (solid square) were too small to be fitted, indicating that no binding was observed in the conditions used (G and H).

crowding effect, compared with the results of simple crowding calculations as described by Minton [46].

Ficoll 70 and dextran 70 are widely used to mimic the excluded-volume effects in crowded physiological environments [3,33]. Compared with dextran 70, Ficoll 70 behaves much more like a rigid sphere with a radius ~55 Å [10,14]. Ficoll 70 is a highly branched copolymer of two short building blocks, sucrose and epichlorohydrin, making it less flexible and more compact than dextran 70 on a molecular weight basis. In contrast, dextran 70, a flexible, long-chain poly(D-glucose) with sparse, short branches, is better modeled as a rod-like particle [10,14]. Both dextran 70 and Ficoll 70 are branched synthetic polymers. They will interpenetrate each other above overlap concentration, which is <100 g/L [47]. In the present study, we found that the enhancing effect of dextran 70 on the structural stability of apo-HLA was only slightly stronger than that of Ficoll 70. Although Ficoll 70 is more compact than dextran 70, both synthetic polymers have similar excluded volumes under this situation.

In contrast with Ficoll 70 and dextran 70, PEG is another kind of crowding agent, whose interactions with proteins can be described as a competition between a repulsive excluded volume interaction between PEG and proteins and an attractive interaction between PEG and proteins [3,10]. It would be worthwhile to extend the current work to analyze the effects of high concentrations of ethylene glycol, sucrose, or glucose, which are the 'monomers' of PEG, Ficoll, or dextran. These are planned for the future. Both Ficoll 70 and dextran 70 are 70 kDa, but PEG 2000 is only 2 kDa. It has been reported that small solutes destabilize proteins and larger solutes stabilize proteins [48]. Recently, it has been shown that small PEGs actually destabilize a 12-nucleotide DNA strand, while larger PEGs stabilize DNA [49]. Therefore, we further used PEG 20000 as the crowding agent. Our data indicated that both Ficoll 70 and dextran 70 enhanced the structural stability of apo-HLA represented by the transition to a molten globule state but PEG 2000 and PEG 20000 significantly decreased the structural stability of apo-HLA. Furthermore, no interaction between apo-HLA and Ficoll 70 or dextran 70 but a weak, non-specific interaction between the apo form of the protein and PEG 2000 was detected by ITC. Our control ITC experiments with high salt showed no interaction between apo-HLA and PEG 2000 in the presence of 1.0 M NaCl (data not shown), demonstrating that high salt can eliminate non-specific interactions. Recently, it has been suggested that the dominant factors affecting protein

behavior *in vivo* are a combination of excluded volume effects and weak attractive forces [10,15,17–19,50,51]. We thus suggest that such an attractive interaction between PEG and apo-HLA could overcome the repulsive excluded volume interaction between PEG and apo-HLA, thereby decreasing the structural stability of apo-HLA. Our data are consistent with the results of protein stability studies in cells [18,52] and suggest that stabilizing excluded-volume effects of crowding agents can be ameliorated by non-specific interactions between non-native forms of proteins and crowders. The interior of a typical cell is highly crowded with proteins, nucleic acids, and polysaccharides, in which proteins must jostle and compete with each other in order to carry out their biological functions [52]. The heterogeneity of the crowded physiological environment can create regions where proteins are stabilized or destabilized, depending on the local degree of volume exclusion and the local extent of non-specific interactions [15,18,19]. Therefore, we propose that in crowded physiological environments, different crowding agents have different effects on the structural stability of nearby proteins, and thereby regulate their biological functions in a subtle but important way.

Acknowledgement

The authors would like to thank Prof. Guang-Fu Yang (College of Chemistry, Central China Normal University) for his technical assistance on iTC_{200}.

Funding

This work was supported by grants from the National Key Basic Research Foundation of China (2012CB911003), the National Natural Science Foundation of China (30970599 and 31170744), and the Fundamental Research Funds for the Central Universities of China (1104006).

References

1 Zimmerman SB and Minton AP. Macromolecular crowding: biochemical, biophysical, and physiological consequences. Annu Rev Biophys Biomol Struct 1993, 22: 27–65.
2 Minton AP. How can biochemical reactions within cells differ from those in test tubes? J Cell Sci 2006, 119: 2863–2869.
3 Zhou HX, Rivas G and Minton AP. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. Annu Rev Biophys 2008, 37: 375–397.
4 Minton AP. Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations. J Pharmacol Sci 2005, 94: 1668–1675.
5 Charlton LM, Barnes CO, Li C, Orans J, Young GB and Pielak GJ. Residue-level interrogation of macromolecular crowding effects on protein stability. J Am Chem Soc 2008, 130: 6826–6830.
6 Sasahara K, McPhie P and Minton AP. Effect of dextran on protein stability and conformation attributed to macromolecular crowding. J Mol Biol 2003, 326: 1227–1237.
7 Zhou BR, Liang Y, Du F, Zhou Z and Chen J. Mixed macromolecular crowding accelerates the oxidative refolding of reduced, denatured lysozyme: implications for protein folding in intracellular environments. J Biol Chem 2004, 279: 55109–55116.
8 Tokuriki N, Kinjo M, Negi S, Hoshino M, Goto Y, Urabe I and Yomo T. Protein folding by the effects of macromolecular crowding. Protein Sci 2004, 13: 125–133.
9 Spencer DS, Xu K, Logan TM and Zhou HX. Effects of pH, salt, and macromolecular crowding on the stability of FK506-binding protein: an integrated experimental and theoretical study. J Mol Biol 2005, 351: 219–232.
10 Du F, Zhou Z, Mo ZY, Shi JZ, Chen J and Liang Y. Mixed macromolecular crowding accelerates the refolding of rabbit muscle creatine kinase: implications for protein folding in physiological environments. J Mol Biol 2006, 364: 469–482.
11 Stagg L, Zhang SQ, Cheung MS and Stafshede PW. Molecular crowding enhances native structure and stability of α/β protein flavodoxin. Proc Natl Acad Sci USA 2007, 104: 18976–18981.
12 McPhie P, Ni Y and Minton AP. Macromolecular crowding stabilizes the molten globule form of apomyoglobin with respect to both cold and heat unfolding. J Mol Biol 2006, 361: 7–10.
13 Yuan JM, Chyan CL, Zhou HX, Chung TY, Peng H, Ping G and Yang G. The effects of macromolecular crowding on the mechanical stability of protein molecules. Protein Sci 2008, 17: 2156–2166.
14 Christiansen A, Wang Q, Samiotakis A, Cheung MS and Wittung-Stafshede P. Factors defining effects of macromolecular crowding on protein stability: an in vitro/in silico case study using cytochrome c. Biochemistry 2010, 49: 6519–6530.
15 Miklos AC, Li C, Sharaf NG and Pielak GJ. Volume exclusion and soft interaction effects on protein stability under crowded conditions. Biochemistry 2010, 49: 6984–6991.
16 Hong J and Gierasch LM. Macromolecular crowding remodels the energy landscape of a protein by favoring a more compact unfolded state. J Am Chem Soc 2010, 132: 10445–10452.
17 Aguilar X, Weise CF, Sparrman T, Watz MW and Stafshede PW. Macromolecular crowding extended to a heptameric system: the co-chaperonin protein 10. Biochemistry 2011, 50: 3034–3044.
18 Schlesinger AP, Wang Y, Tadeo X, Millet O and Pielak GJ. Macromolecular crowding fails to fold a globular protein in cells. J Am Chem Soc 2011, 133: 8082–8085.
19 Miklos AC, Sarkar M, Wang Y and Pielak GJ. Protein crowding tunes protein stability. J Am Chem Soc 2011, 133: 7116–7120.
20 Ellis RJ and Minton AP. Protein aggregation in crowded environments. Biol Chem 2006, 387: 485–497.
21 Minton AP. Implications of macromolecular crowding for protein assembly. Curr Opin Struct Biol 2000, 10: 34–39.
22 Kuwajima K. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. Proteins 1989, 6: 87–103.
23 Kuwajima K. The molten globule state of α-lactalbumin. FASEB J 1996, 10: 102–109.
24 Permyakov EA and Berliner LJ. α-Lactalbumin: structure and function. FEBS Lett 2000, 473: 269–274.
25 Ramboarina S and Redfield C. Structural characterisation of the human α-lactalbumin molten globule at high temperature. J Mol Biol 2003, 330: 1177–1188.

26 Kataoka M, Kuwajima K, Tokunaga F and Goto Y. Structural characterization of the molten globule of α-lactalbumin by solution X-ray scattering. Protein Sci 1997, 6: 422–430.

27 Troullier A, Reinstadler D, Dupont Y, Naumann D and Forge V. Transient non-native secondary structures during the refolding of α-lactalbumin detected by infrared spectroscopy. Nat Struct Biol 2000, 7: 78–86.

28 Mok KH, Nagashima T, Day LJ, Hore PJ and Dobson CM. Multiple subsets of side-chain packing in partially folded states of α-lactalbumins. Proc Natl Acad Sci USA 2005, 102: 8899–8904.

29 Yang F, Jr, Zhang M, Chen J and Liang Y. Structural changes of α-lactalbumin induced by low pH and oleic acid. Biochim Biophys Acta 2006, 1764: 1389–1396.

30 Svensson M, Hakansson A, Mossberg AK, Linse S and Svanborg C. Conversion of α-lactalbumin to a protein inducing apoptosis. Proc Natl Acad Sci USA 2000, 97: 4221–4226.

31 Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg A and Svanborg C. Treatment of skin papillomas with topical α-lactalbumin-oleic acid. N Engl J Med 2004, 350: 2663–2672.

32 Zhang M, Yang F, Jr, Yang F, Chen J, Zheng CY and Liang Y. Cytotoxic aggregates of α-lactalbumin induced by unsaturated fatty acid induce apoptosis in tumor cells. Chem Biol Interact 2009, 180: 131–142.

33 Zhou Z, Yan X, Pan K, Chen J, Xie ZS, Xiao GF and Yang FQ, *et al*. Fibril formation of the rabbit/human/bovine prion proteins. Biophys J 2011, 101: 1483–1492.

34 Kaplanas RI and Antanavichyus AI. Isolation and purification of α-lactalbumin, a component of lactose synthetase. Biochemistry 1975, 40: 493–495.

35 Nozaka M, Kuwajima K, Nitta K and Sugai S. Detection and characterization of the intermediate on the folding pathway of human α-lactalbumin. Biochemistry 1978, 17: 3753–3758.

36 Permyakov SE, Pershikova IV, Zhadan AP, Goers J, Bakunts AG, Uversky VN and Berliner LJ, *et al*. Conversion of human α-lactalbumin to an apo-like state in the complexes with basic poly-amino acids: toward understanding of the molecular mechanism of antitumor action of HAMLET. J Proteome Res 2005, 4: 564–569.

37 Stănciuc N, Râpeanu G, Bahrim G and Aprodu I. pH and heat-induced structural changes of bovine apo-α-lactalbumin. Food Chem 2012, 131: 956–963.

38 Liang Y. Applications of isothermal titration calorimetry in protein science. Acta Biochim Biophys Sin 2008, 40: 565–576.

39 Liang Y, Du F, Sanglier S, Zhou BR, Xia Y, Van Dorsselaer A and Maechling C, *et al*. Unfolding of rabbit muscle creatine kinase induced by acid. A study using electrospray ionization mass spectrometry, isothermal titration calorimetry, and fluorescence spectroscopy. J Biol Chem 2003, 278: 30098–30105.

40 Mo ZY, Zhu YZ, Zhu HL, Fan JB, Chen J and Liang Y. Low micromolar zinc accelerates the fibrillization of human Tau via bridging of Cys-291 and Cys-322. J Biol Chem 2009, 284: 34648–34657.

41 Zhu HL, Fernández C, Fan JB, Shewmaker F, Chen J, Minton AP and Liang Y. Quantitative characterization of heparin binding to Tau protein. Implication for inducer-mediated Tau filament formation. J Biol Chem 2010, 285: 3592–3599.

42 Wu LJ, Xu LR, Liao JM, Chen J and Liang Y. Both the C-terminal polylysine region and the farnesylation of K-RasB are important for its specific interaction with calmodulin. PLoS One 2011, 6: e21929.

43 Zhu HL, Meng SR, Fan JB, Chen J and Liang Y. Fibrillization of human Tau is accelerated by exposure to lead via interaction with His-330 and His-362. PLoS One 2011, 6: e25020.

44 Chrysina ED, Brew K and Acharya KR. Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2-Å resolution reveal an effect of calcium on inter-lobe interactions. J Biol Chem 2000, 275: 37021–37029.

45 Xie D, Bhakuni V and Freire E. Calorimetric determination of the energetics of the molten globule intermediate in protein folding: apo-α-lactalbumin. Biochemistry 1991, 30: 10673–10678.

46 Minton AP. Excluded volume as a determinant of macromolecular structure and reactivity. Biopolymers 1981, 20: 2093–2120.

47 Rubinstein M and Colby RH. Polymer Physics. England: Oxford University Press, 2003.

48 Zhou Y and Hall CK. Solute excluded-volume effects on the stability of globular proteins: A statistical thermodynamic theory. Biopolymers 1996, 38: 273–284.

49 Knowles DB, LaCroix AS, Deines NF, Shkel I and Record MT, Jr. Separation of preferential interaction and excluded volume effects on DNA duplex and hairpin stability. Proc Natl Acad Sci USA 2011, 108: 12699–12704.

50 Jiao M, Li HT, Chen J, Minton AP and Liang Y. Attractive protein-polymer interactions markedly alter the effect of macromolecular crowding on protein association equilibria. Biophys J 2010, 99: 914–923.

51 Li C and Pielak GJ. Using NMR to distinguish viscosity effects from nonspecific protein binding under crowded conditions. J Am Chem Soc 2009, 131: 1368–1369.

52 McGuffee SR and Elcock AH. Diffusion, Crowding & protein stability in a dynamic molecular model of the bacterial cytoplasm. PLoS Comput Biol 2010, 6: e1000694.